MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME

Field of the Invention

The present invention relates to a modified human granulocyte-colony stimulating factor(hG-CSF), a gene encoding said peptide, a vector comprising said gene, a microorganism transformed with said vector and a process for producing the modified hG-CSF using said microorganism.

Background of the Invention

The term colony stimulating factor (CSF) is inclusive of granulocyte/macrophage-colony stimulating factor(GM-CSF), macrophage-colony stimulating factor(M-CSF) and granulocyte-colony stimulating factor(G-CSF), which are produced by T-cells, macrophages, fibroblasts and endothelial cells. GM-CSF stimulates stem cells of granulocyte or macrophage to induce the differentiation thereof and proliferation of granulocyte or macrophage colonies. M-CSF and G-CSF primarily induce the formation of the colonies of macrophage and granulocyte, respectively. In vivo, G-CSF induces the differentiation of bone marrow leucocytes and enhances the function of mature granulocyte and, accordingly, it's clinical importance in treating leukemia has been well established.

Human G-CSF(hG-CSF) is a protein consisting of 174 or 177 amino acids, the 174 amino-acid variety having higher neutrophil-enhancing activity(Morishita, K. et al., J. Biol. Chem., 262, 15208-15213(1987)). The amino acid sequence of hG-CSF consisting of 174 amino acids is shown in Fig. 1 and there have been many studies for the mass production of hG-CSF by manipulating a gene encoding said hG-CSF.

For instance, Chugai Pharmaceuticals Co., Ltd.(Japan) has disclosed the amino acid sequence of hG-CSF and a gene encoding same(Korean Patent Publication Nos. 91-5624 and 92-2312), and reported a method for preparing proteins having hG-CSF activity by a gene recombination process(Korean Patent Nos. 47178, 53723 and 57582). In this preparation method, glycosylated hG-CSF is produced in a mammalian cell by employing a genomic DNA or cDNA comprising a polynucleotide encoding hG-CSF. The glycosylated hG-

secretory signal peptide prepared by modifying the signal peptide of *E. coli* thermoresistant enterotoxin II(Korean Patent Laid-open publication No. 2000-19788) in the production of hG-CSF. Specifically, an expression vector comprising a hG-CSF gene attached to the 3'-end of the modified signal peptide of *E. coli* thermoresistant enterotoxin II was prepared, and biologically active, mature hG-CSF was expressed by employing *E. coli* transformed with the expression vector. However, most of the expressed hG-CSF accumulated in the cytoplasm rather than in the periplasm.

The present inventors have endeavored further to develop an efficient secretory method for the production of hG-CSF in a microorganism and have found that a modified hG-CSF, which is prepared by replacing at least one amino acid residue, especially, the 17th cysteine residue, of wild-type hG-CSF with other amino acid, retains the biological activity of the wild-type, and that the modified hG-CSF having no methionine residue at the N-terminus thereof can be efficiently expressed and secreted by a microorganism when an appropriate secretory signal peptide is employed.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a modified human granulocyte-stimulating factor(hG-CSF) which can be efficiently produced using a microorganism..

It is another object of the present invention to provide a gene encoding said peptide and a vector comprising said gene.

It is a further object of the present invention to provide a microorganism transformed with said vector.

It is a still further object of the present invention to provide a process for producing a hG-CGF which is non-attached methionine residue to amino terminus using said microorganism.

In accordance with one aspect of the present invention, there is provided a modified hG-CSF characterized in that at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF is replaced by another amino acid.

(d) the 2nd amino acid is Met, the 3rd amino acid is Val and the 17th amino acid is X; or

(f) the 17th amino acid is X,

wherein X is an amino acid which is not charged at neutral pH., preferably Ser, Thr, Ala or Gly, more preferably Ser.

Four of the five Cys residues of hG-CSF participate in forming disulfide bonds, while the 17th Cys residue remains unbonded in the natural state. However, when a large amount of hG-CSF is expressed in recombinant cells, the 17th Cys residue gets involved in inter-molecular disulfide bond formation, leading to the accumulation of agglomerated hG-CSFs in the cytoplasm. However, the inventive modified hG-CSF having an amino acid other than Cys at the 17th position is free of such problem and can be effectively produced by a secretory method using an appropriately transformed microorganism.

The modified hG-CSF of the present invention may be encoded by a gene comprising a nucleotide sequence deduced from the modified hG-CSF amino acid sequence according to the genetic code. It is known that several different codons encoding a specific amino acid may exist due to the codon degeneracy, and, therefore, the present invention includes in its scope all nucleotide sequences deduced from the modified hG-CSF amino acid sequence. Preferably, the modified hG-CSF gene sequence includes one or more preferred codons of *E. coli*.

The gene thus prepared may be inserted to a conventional vector to obtain an expression vector, which may, in turn, be introduced into a suitable host, e.g., an *E. coli*. The expression vector may further comprise a signal peptide. Representative signal peptides include a thermoresistant *E. coli*. enterotoxin II signal peptide(SEQ ID NO: 53), a modified thermoresistant *E. coli* enterotoxin II signal peptide(SEQ ID NO: 54), a beta lactamase signal peptide(SEQ ID NO: 24), Gene III signal peptide(SEQ ID NO: 42) or modified peptide thereof, but these do not limit the signal peptides which may be used in the present invention. The promoter used in preparing the expression vector of present invention may be any of those which can express a heterologous protein in a microorganism host. Specially, lac, Tac, and arabinose promoter is preferred when the heterologous protein is expressed from *E. coli*.

Exemplary expression vector of the present invention includes

solution may be conducted by a conventional method(Sambrook et al., the supra), e.g., ion exchange chromatography, gel filtration column chromatography or immune column chromatography. For example, hG-CSF may be purified by sequentially conducting CM-Sepharose column chromatograph and Phenyl Sepharose column chromatography.

The modified hG-CSF protein produced according to the present invention is not methionylated at the N-terminus and has biological activity which is equal to, or higher than, that of wild-type hG-CSF. Therefore, it may be used as is in various applications

The following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1: Preparation of A Gene Encoding hG-CSF

A cDNA gene encoding hG-CSF was prepared by carrying out PCR using as an hG-CSF template(R&D system, USA). The primers used are those described in US patent No. 4,810,643.

To prepare a cDNA gene encoding mature hG-CSF, vector pUC19-G-CSF(Biolabs, USA) was subjected to PCR using the primers of SEQ ID NOS: 3 and 4. The primer of SEQ ID NO: 3 was designed to provide an NdeI restriction site(5'-CATATG-3') upstream from the first amino acid(threonine) codon of mature hG-CSF, and the primer of SEQ ID NO: 4, to provide a BamHI restriction site(5'-GGATCC-3') downstream from the termination codon thereof.

The amplified hG-CSF gene was cleaved with NdeI and BamHI to obtain a gene encoding mature hG-CSF. The hG-CSF gene was inserted at the NdeI/BamHI section of vector pET14b(Novagen, USA) to obtain vector pT-CSF.

Fig. 2 shows the above procedure for constructing vector pT-CSF.

Example 2: Construction of a vector containing the gene encoding *E. coli* enterotoxin II signal peptide and a modified hG-CSF

(Step 1) Cloning *E. coli* enterotoxin II signal peptide gene

To prepare *E. coli* enterotoxin II signal peptide gene, the pair of complementary oligonucleotides having SEQ ID NOS: 5 and 6 were designed

primer of SEQ ID NO: 10, to provide a BamHI restriction site downstream from the termination codon of mature hG-CSF to obtain a DNA fragment(STII SD-STII-hCSF) containing a STII SD and STII-hG-CSF gene.

The STII SD-STII-hG-CSF fragment was cleaved with XbaI and BamHI, and then inserted at the XbaI/BamHI section of vector pET14b(Novagen, USA) to obtain vector pT14SS1SG.

Fig. 4 displays the above procedure for constructing vector pT14SS1SG.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pT14SS1SG to obtain a transformant designated *E. coli* HM 10310.

(Step 4) Construction of a vector containing a gene encoding STII/hG-CSF fusion protein

The first codon of the modified hG-CSF gene of plasmid pT14SS1SSG obtained in Step 3 was replaced by Thr in accordance with a site-directed mutagenesis(Papworth, C. et al., Strategies, 9, 3(1996)), which was conducted by PCR of the plasmid with a sense primer(SEQ ID NO: 12) having a modified nucleotide sequence, a complementary antisense primer(SEQ ID NO: 13), and pfu(Stragene, USA).

The amplified DNA fragment was recovered and restriction enzyme DpnI was added thereto to remove unconverted plasmids.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pT14SSG which contained a gene having Thr in place of the first amino acid of hG-CSF(SEQ ID NO: 11).

```
 -5  -4  -3  -2  -1  +1  +2  +3  +4  +5
 Thr Asn Ala Tyr Ala Thr Pro Leu Gly Pro   (SEQ ID NO: 11)
- ACA-AAT-GCC-TAC-GCG-ACA-CCC-CTG-GGC-CCT  (SEQ ID NO: 12)
- TGT-TTA-CGG-ATG-CGC-TGT-GGG-GAC-CCG-GGA  (SEQ ID NO: 13)
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E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pT14SSG to obtain a transformant designated *E. coli* HM 10301.

(Step 7) Construction of a vector containing a modified STII SD and a gene encoding modified STII /hG-CSF

Vector pT14SSG-4T22Q obtained in Step 6 was subjected to PCR using the complementary primers of SEQ ID NOS: 20 and 21 in accordance with the procedure of Step 4 to obtain vector pT140SSG-4T22Q having the six nucleotide sequences between the STII SD sequence(GAGG) and the initiation codon of STII(modified STII SD of SEQ ID NO: 71).

Fig. 5 represents the above procedure for constructing vector pT140SSG-4T22Q.

E. coli BL21(DE3) was transformed with vector pT140SSG-4T22Q to obtain a transformant designated *E. coli* HM 10302.

Example 3: Construction of a vector containing a gene encoding modified hG-CSF

To prepare a modified hG-CSF gene, S1 oligomer(SEQ ID NO: 22) having *E. coli*-preferred codons and Ser in place of the 17th amino acid of hG-CSF and AS1 oligomer(SEQ ID NO: 23) were synthesized using DNA synthesizer(Model 380B, Applied Biosystem, USA).

0.5 $\mu\ell$(50 pmole) quantities of the oligonucleotides were reacted at 95 ℃ for 15 min. and kept until 35 ℃ for 3 hours. The mixture was precipitated in ethanol and subjected to gel electrophoresis(SDS-PAGE) to obtain a cohesive ended double strand(ds) oligomer.

The plasmid pT14SS1SG obtained in step 3 of Example 2 was cleaved with ApaI and BstXI, and then ligated with the adhesive-ended ds oligomer, to obtain vector pT14SS1S17SEG. Vector pT14SS1S17SEG contained a gene encoding hG-CSF having *E. coli*-preferred codons at the amino terminus and Ser in place of the 1st and 17th amino acids of hG-CSF, respectively.

Fig. 6 illustrates the above procedure for constructing vector pT140SS1S17SEG.

E. coli BL21(DE3) was transformed with vector pT14SS1S17SEG to obtain a transformant designated *E. coli* HM 10311, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number KCCM-10154.

plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained plasmid pTOG which contained a gene having Thr in place of the first amino acid of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTOG to obtain a transformant designated *E. coli* HM 10401.

Example 6: Production of modified hG-CSFs

(a) Production of [Ser1, Ser17] hG-CSF

Vector pTO1SG obtained in Example 4 was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined and thus obtained was plasmid pTO1S17SG which contained a gene having Ser in place of the 1st and 17th amino acids of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO1S17SG to obtain a transformant designated *E. coli* HM 10410, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number KCCM-10151.

(b) Production of [Ser17] hG-CSF

Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17SG which contained a gene having Ser in place of the 17th amino acid of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17SG to obtain a transformant designated *E. coli* HM 10411, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24,

was determined, and thus obtained was plasmid pTO17GG which contained a gene having Gly in place of the 17th amino acids of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17GG to obtain a transformant designated *E. coli* HM 10415.

(f) Production of [Asp17] hG-CSF

Vector pTOG obtained in Example 5 was subjected to PCR using a sense primer(SEQ ID NO: 40) designed to substitute Asp codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 41) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pTO17APG which contained a gene having Asp in place of the 17th amino acids of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pTO17APG to obtain a transformant designated *E. coli* HM 10416.

Example 7: Construction of a vector containing a gene encoding *E. coli* Gene III signal peptide and modified hG-CSF

(a) Construction of a vector containing a gene encoding arabinose promoter and *E. coli* Gene III signal peptide

A vector containing a gene encoding arabinose promoter and *E. coli* Gene III signal peptide(SEQ ID NO: 42) as well as a gene encoding modified hG-CSG was prepared as follows:

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Met-Lys-Lys-Leu-Leu-Phe-Ala-Ile-Pro-Leu-Val-Val-Pro-
Phe-Tyr-Ser-His-Ser-                          (SEQ ID NO: 42)
   -TAT-AGC-CAT-AGC-ACC-ATG-GAG-              (SEQ ID NO: 43)
   -ATA-TCG-GTA-TCG-TGG-TAC-CTC-              (SEQ ID NO: 44)
                  NcoI restriction site
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Plasmid pBAD · gIIIA(Invitrogen, USA) containing a gene encoding arabinose promoter and Gene III signal peptide was cleaved with NcoI, and

Thr Met Val Gly Pro Ala Ser Ser Leu (SEQ ID NO: 49)
5'-TAC-GCG-TCC-ATG-GTG-GGC-CCT-GCC-AGC-TCC-CTG-3'(SEQ ID NO: 50)
3'-ATG-CGC-AGG-TAC-CAC-CCG-GGA-CGG-TCG-AGG-GAC-5'(SEQ ID NO: 51)
NcoI restriction site

The hG-CSF gene fragment was inserted into the vector obtained above to obtain vector pBAD2M2VG contained a gene coding *E. coli* Gene III signal peptide, and Met and Val in place of the 2nd and 3rd amino acids of hG-CSF(SEQ ID NO: 52), respectively.

Fig. 9 shows the above procedure for constructing vector pBAD2M3VG.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pBAD2M3VG to obtain a transformant designated *E. coli* HM 10510, which was deposited with Korean Culture Center of Microorganisms(KCCM) on March 24, 1999 under accession number of KCCM-10153.

(c) Production of [Ser17] hG-CSF

Vector pBADG obtained in (a) was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified plasmid.

E. coli XL-1 blue(Novagen, USA) was transformed with the modified plasmid. The base sequence of the DNA recovered from transformed colonies was determined, and thus obtained was plasmid pBAD17SG which contained a gene having Ser in place of the 17th amino acid of hG-CSF.

E. coli BL21(DE3)(Stratagene, USA) was transformed with vector pBAD17SG to obtain a transformant designated *E. coli* HM 10511.

(d) Production of [Met2, Val3, Ser17] hG-CSF

Vector pBAD2M3VG obtained in (b) was subjected to PCR using a sense primer(SEQ ID NO: 32) designed to substitute Ser codon for the 17th codon of hG-CSF and a complementary antisense primer(SEQ ID NO: 33) in accordance with the procedure of Step 4 of Example 2 to obtain a modified

Table 1

Transformant	Example	Expression Vector	hG-CSF Level in periplasm(mg/ℓ)
HM 10301	2(Step 4)	pT14SSG	65
HM 10302	2(Step 7)	pT140SSG-4T22Q	277
HM 10310	2(Step 3)	pT14SS1SG	92
HM 10311	3	pT14SS1S17SEG	1,512
HM 10401	5	pTOG	85
HM 10409	4	pTO1SG	105
HM 10410	6(a)	pTO1S17SG	1,477
HM 10411	6(b)	pTO17SG	1,550
HM 10413	6(c)	pTO17TG	1,373
HM 10414	6(d)	pTO17AG	1,486
HM 10415	6(e)	pTO17GG	1,480
HM 10416	6(f)	pTO17APG	67
HM 10501	7(a)	pBADG	54
HM 10510	7(b)	pBAD2M3VG	69
HM 10511	7(c)	pBAD17SG	937
HM 10512	7(d)	pBAD2M3V17SG	983

Example 9: Purification of hG-CSF

Transformant *E. coli* HM 10411 prepared in Example 6(b) was cultured in LB medium and the culture was centrifuged for 6,000 rpm for 20 min. to harvest cells. The periplasmic solution was prepared from the cells by repeating the procedure of Example 8.

The periplasmic solution was adjusted to pH 5.0 to 5.5, adsorbed on a CM-Sepharose(Pharmacia Inc., Sweden) column pre-equilibrated to pH 5.3, and then, the column was washed with 25 mM NaCl. hG-CSF was eluted by sequentially adding to the column buffer solutions containing 50mM, 100mM and 200mM NaCl, and fractions containing hG-SCF were collected and combined.

The combined fractions were subjected to Phenyl Sepharose(Pharmacia Inc., Sweden) column chromatography to obtain [Ser17] hG-CSF having a purity of 99%.

and lane 2, purified [Ser17] hG-CSF. As can be seen from Fig. 10b, the molecular weight of [Ser17] hG-CSF equals that of wild-type hG-CSF.

Example 10: Cellular Activity of hG-CSF and Modified hG-CSF

Cell line HL-60(ATCC CCL-240 derived from the bone marrow of a promyelocytic leukemia patient/a white 36-year-old woman) was cultured in RPMI 1640 media containing 10% fetal bovine serum and adjusted to 2.2 X 10^5 cells/mℓ, followed by adding thereto DMSO(dimethylsulfoxide, culture grade/SIGMA) to a concentration of 1.25%(v/v). 90 μℓ of the resulting solution was added to a 96 well plate(Corning/low evaporation 96 well plate) in an amount of 2 X 10^4 cells/well and incubated at 37℃ under 5% CO2 for 48 hours.

Each of the modified [Ala17] hG-CSF, [Gly 17] hG-CSF, [Ser17] hG-CSF, and [Thr 17] hG-CSF was diluted in RPMI 1640 media to a concentration of 500 ng/mℓ and then serially diluted 10 times by 2-fold with RPMI 1640 media.

The resulting solution was added to wells at 10 μℓ per well and incubated at 37 ℃ for 48 hours. As a positive control, a commercially available hG-CSF(Jeil Pharmaceutical.).

The level of cell line increased was determined using a commercially available CellTiter96™(Cat # G4100, Promega) based on the measured optical density at 670 nm.

As can be seen from Fig. 11, the cellular activities of the modified hG-CSFs are the same as, or higher than of that the positive control, wild-type hG-CSF.

While the embodiments of the subject invention have been described and illustrated, it is obvious that various changes and modifications can be made therein without departing from the spirit of the present invention which should be limited only by the scope of the appended claims.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Hanmi Pharm. Co., Ltd
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KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : *HM10410*	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10151
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authouity accepts the microorganism identified under I above, which was received by it on March. 24. 1999 (date of the original deposit) [1]	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April. 6. 1999

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of i nternational depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authouity.

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Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: April. 6. 1999

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E. coli thermoresistant enterotoxin II signal peptide or modified *E. coli* thermoresistant enterotoxin II signal peptide.

10. The expression vector of claim 9, wherein the *E. coli* thermoresistant enterotoxin II signal peptide has the amino acid sequence of SEQ ID NO: 53.

11. The expression vector of claim 9, wherein the modified *E. coli* thermoresistant enterotoxin II signal peptide has the amino acid sequence of SEQ ID NO: 54.

12. The expression vector of claim 9, which further comprises a modified *E. coli* enterotoxin II Shine-Dalgano sequence having the nucleotide sequence of SEQ ID NO: 71.

13. The expression vector of claim 8, wherein the signal peptide is *E. coli* beta lactamase signal peptide or modified *E. coli* beta lactamase signal peptide.

14. The expression vector of claim 13, wherein the *E. coli* beta lactamase signal peptide has the amino acid sequence of SEQ ID NO: 24.

15. The expression vector of claim 8, wherein the signal peptide is *E. coli* Gene III signal peptide or modified *E. coli* Gene III signal peptide.

16. The expression vector of claim 15, wherein the *E. coli* Gene III signal peptide has the amino acid sequence of SEQ ID NO: 42.

17. The expression vector of claim 7 or 8, which is pT14SS1SG, pT14SS1S17SEG, pTO1SG, pTO1S17SG, pTO17SG or pBAD2M3V17SG.

18. A microorganism transformed with the expression vector according to claim 7 or 8.

19. The microorganism of claim 18, which is a transformed *E. coli*.

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(54) Title: MODIFIED HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR AND PROCESS FOR PRODUCING SAME

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T  P  L  G  P  A  S  S  L  P  Q  S  F  L  L  K
aca ccc ctg ggc cct gcc agc tcc ctg ccc cag agc ttc ctg ctc aag

C  L  E  Q  V  R  K  I  Q  G  D  G  A  A  L  Q
tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag

E  K  L  C  A  T  Y  K  L  C  H  P  E  E  L  V
gag aag ctg tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg gtg

L  L  G  H  S  L  G  I  P  W  A  P  L  S  S  C
ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg agc tcc tgc

P  S  Q  A  L  Q  L  A  G  C  L  S  Q  L  H  S
ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat agc

G  L  F  L  Y  Q  G  L  L  Q  A  L  E  G  I  S
ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg ata tcc

P  E  L  G  P  T  L  D  T  L  Q  L  D  V  A  D
ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc gac

F  A  T  T  I  W  Q  Q  M  E  E  L  G  M  A  P
ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc cct

A  L  Q  P  T  Q  G  A  M  P  A  F  A  S  A  F
gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct ttc

Q  R  R  A  G  G  V  L  V  A  S  H  L  Q  S  F
cag cgc cgg gca gga ggg gtc ctg gtt gct agc cat ctg cag agc ttc

L  E  V  S  Y  R  V  L  R  H  L  A  Q  P
ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcg cag ccc
```

(57) Abstract: A modified human granulocyte-colony stimulating factor (hG-CSF) is produced by culturing a microorganism transformed with an expression vector comprising a gene encoding a modified hG-CSF to produce and secrete the modified hG-CSF to periplasm, said modified hG-CSF being obtained by replacing at least one of the 1st, 2nd, 3rd and 17th amino acids of wild-type hG-CSF (SEQ ID NO: 2) with other amino acid.

WO 01/04329 A1

Fig. 1

```
T  P  L  G  P  A  S  S  L  P  Q  S  F  L  L  K
aca ccc ctg ggc cct gcc agc tcc ctg ccc cag agc ttc ctg ctc aag

C  L  E  Q  V  R  K  I  Q  G  D  G  A  A  L  Q
tgc tta gag caa gtg agg aag atc cag ggc gat ggc gca gcg ctc cag

E  K  L  C  A  T  Y  K  L  C  H  P  E  E  L  V
gag aag ctg tgt gcc acc tac aag ctg tgc cac ccc gag gag ctg gtg

L  L  G  H  S  L  G  I  P  W  A  P  L  S  S  C
ctg ctc gga cac tct ctg ggc atc ccc tgg gct ccc ctg agc tcc tgc

P  S  Q  A  L  Q  L  A  G  C  L  S  Q  L  H  S
ccc agc cag gcc ctg cag ctg gca ggc tgc ttg agc caa ctc cat agc

G  L  F  L  Y  Q  G  L  L  Q  A  L  E  G  I  S
ggc ctt ttc ctc tac cag ggg ctc ctg cag gcc ctg gaa ggg ata tcc

P  E  L  G  P  T  L  D  T  L  Q  L  D  V  A  D
ccc gag ttg ggt ccc acc ttg gac aca ctg cag ctg gac gtc gcc gac

F  A  T  T  I  W  Q  Q  M  E  E  L  G  M  A  P
ttt gcc acc acc atc tgg cag cag atg gaa gaa ctg gga atg gcc cct

A  L  Q  P  T  Q  G  A  M  P  A  F  A  S  A  F
gcc ctg cag ccc acc cag ggt gcc atg ccg gcc ttc gcc tct gct ttc

Q  R  R  A  G  G  V  L  V  A  S  H  L  Q  S  F
cag cgc cgg gca gga ggg gtc ctg gtt gct agc cat ctg cag agc ttc

L  E  V  S  Y  R  V  L  R  H  L  A  Q  P
ctg gag gtg tcg tac cgc gtt cta cgc cac ctt gcg cag ccc
```

Fig. 2

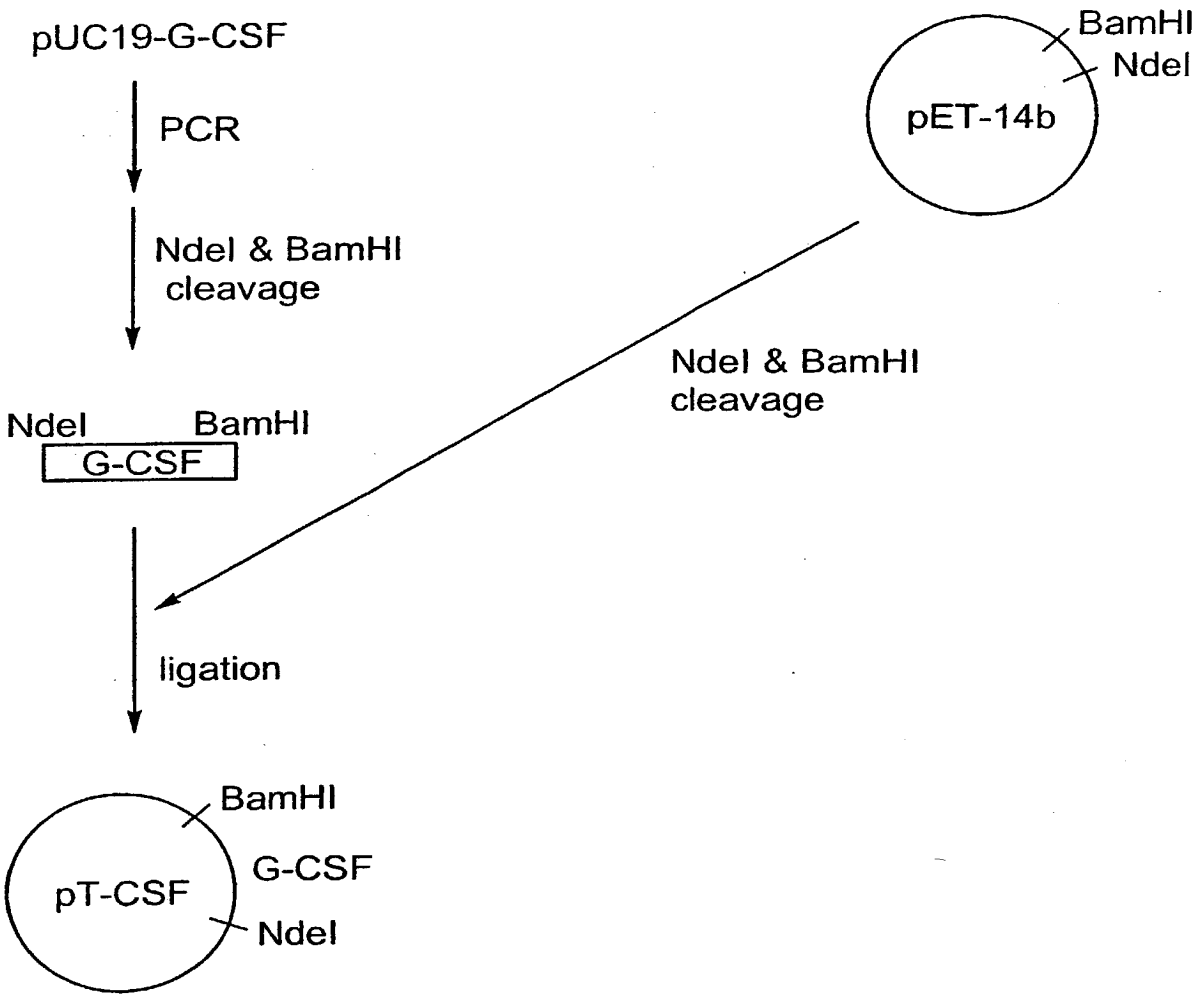

pUC19-G-CSF
PCR
NdeI & BamHI cleavage
NdeI
BamHI
G-CSF
pET-14b
BamHI
NdeI
NdeI & BamHI cleavage
ligation
pT-CSF
BamHI
G-CSF
NdeI

Fig. 3

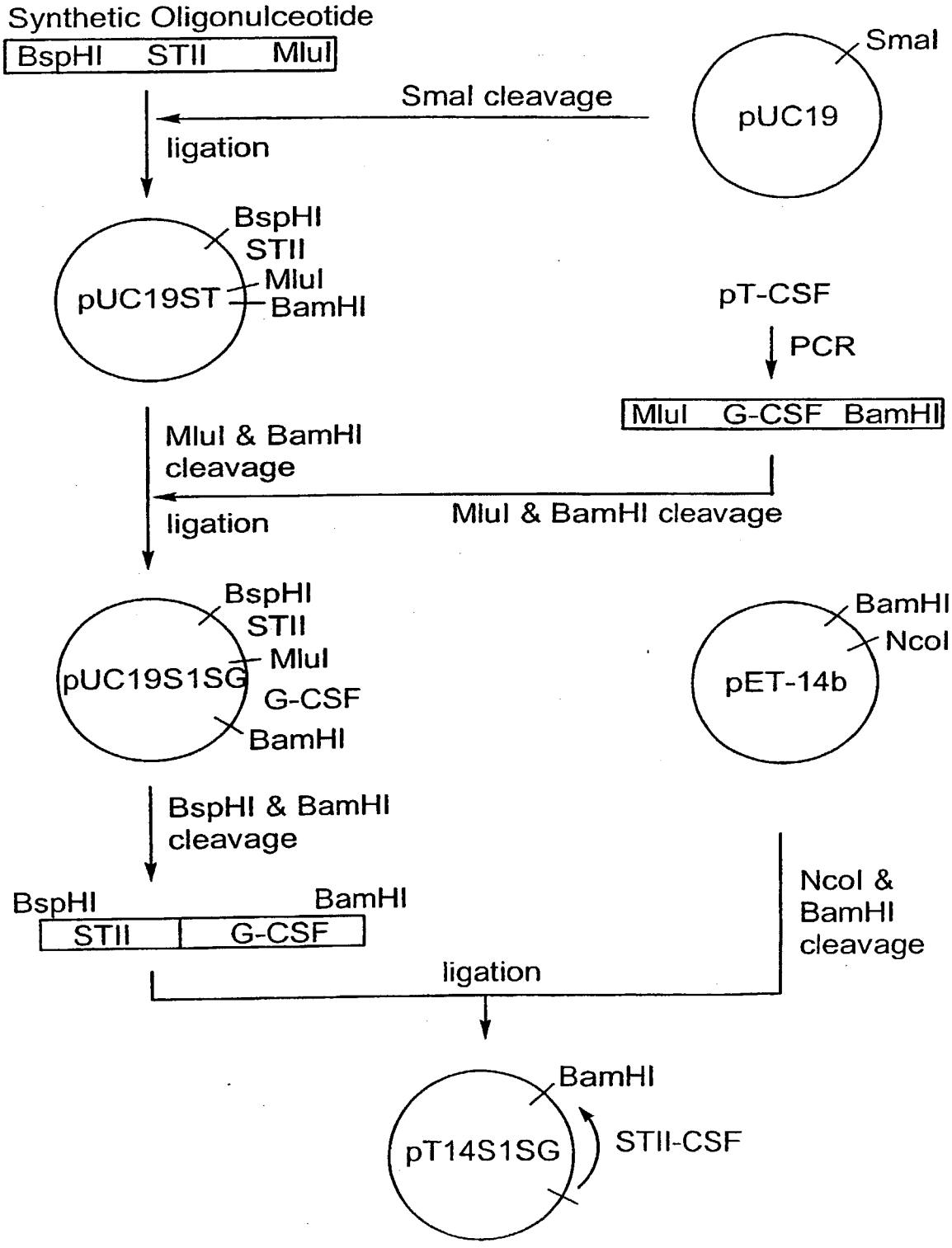

Synthetic Oligonulceotide
BspHI STII MluI
SmaI cleavage
SmaI
pUC19
ligation
BspHI
STII
MluI
pUC19ST
BamHI
pT-CSF
PCR
MluI G-CSF BamHI
MluI & BamHI cleavage
MluI & BamHI cleavage
ligation
BspHI
STII
MluI
pUC19S1SG
G-CSF
BamHI
BamHI
NcoI
pET-14b
BspHI & BamHI cleavage
BspHI
BamHI
STII
G-CSF
NcoI & BamHI cleavage
ligation
BamHI
pT14S1SG
STII-CSF

Fig. 4

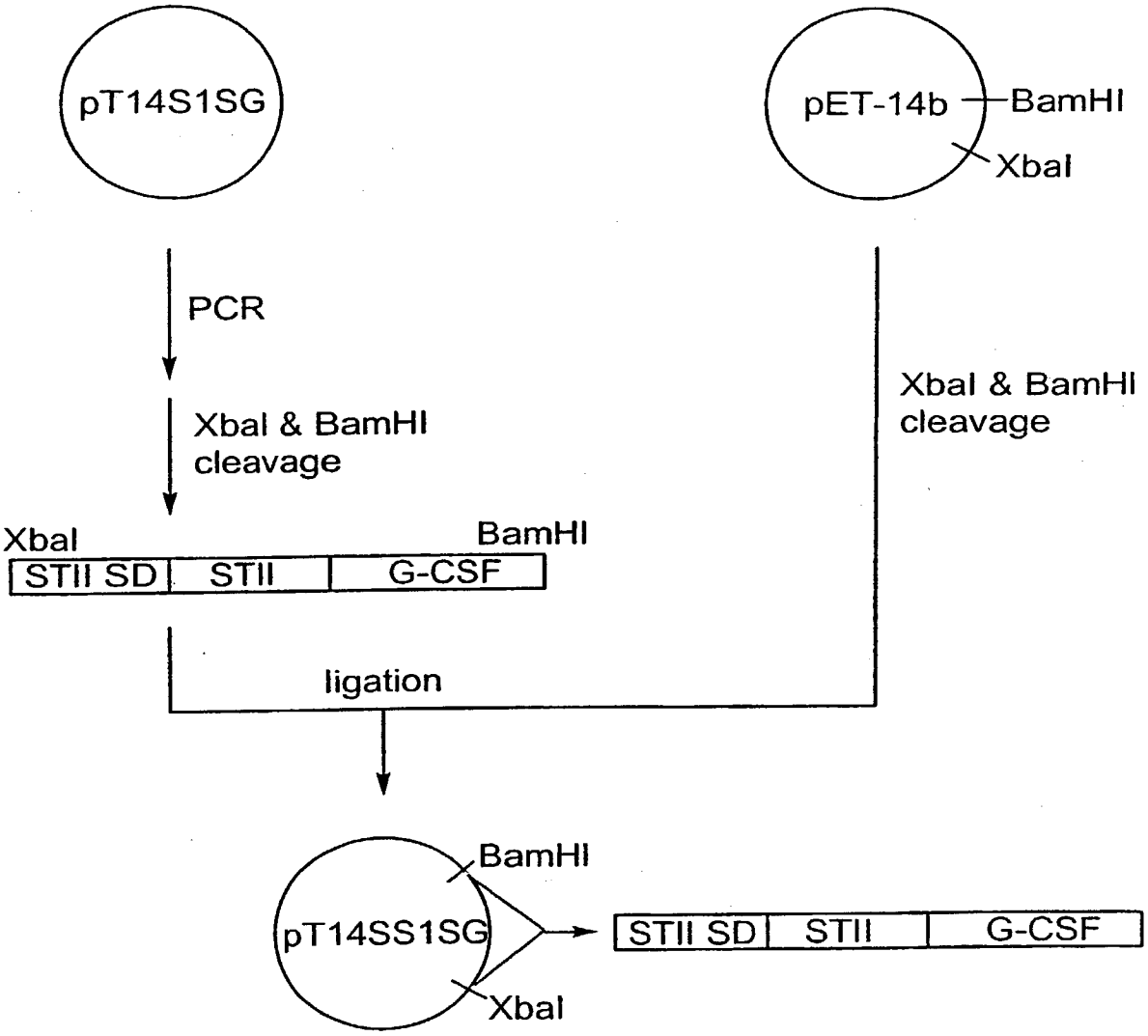

pT14S1SG
pET-14b
BamHI
XbaI
PCR
XbaI & BamHI cleavage
XbaI & BamHI cleavage
XbaI
BamHI
STII SD
STII
G-CSF
ligation
BamHI
pT14SS1SG
STII SD
STII
G-CSF
XbaI

Fig. 5

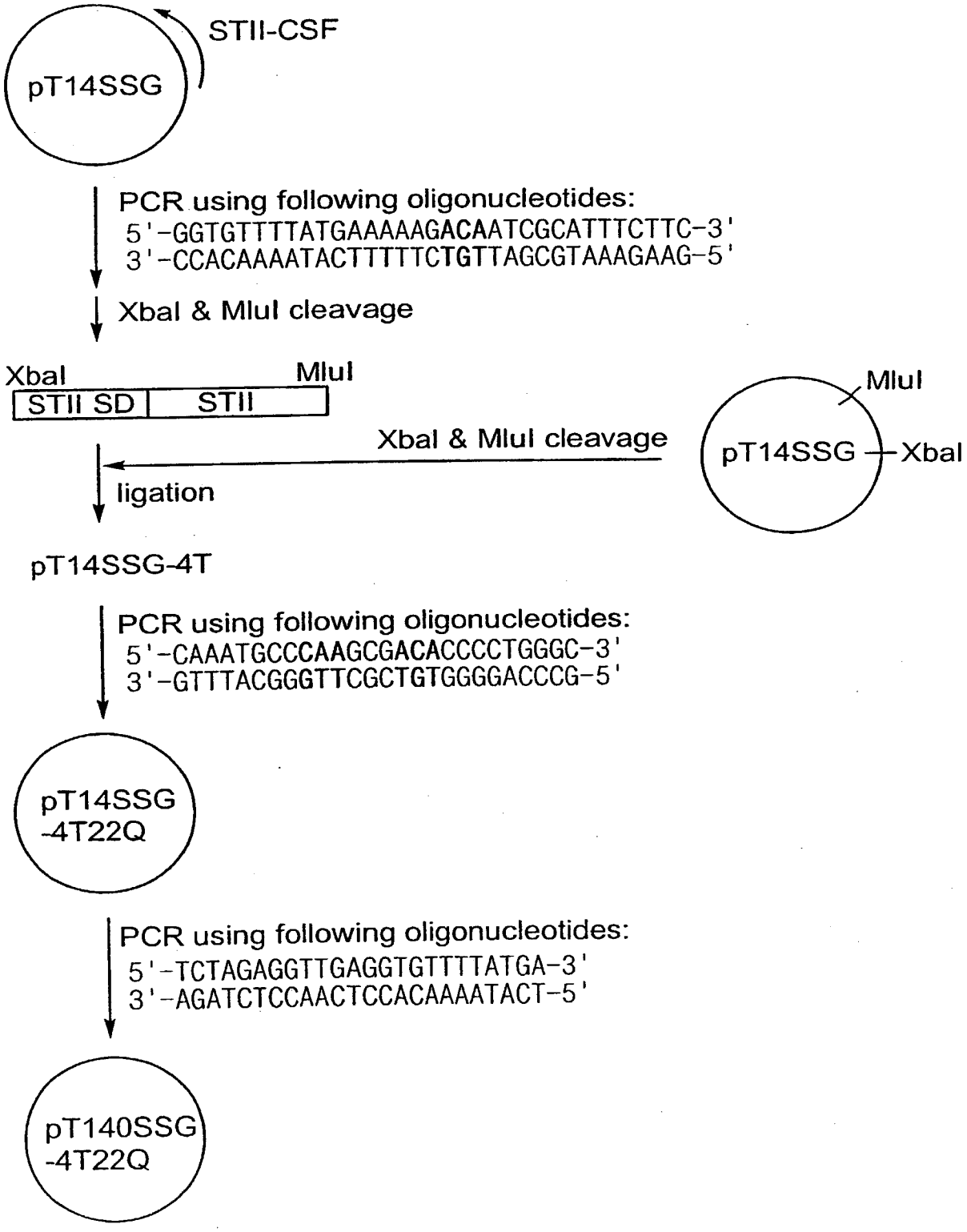

STII-CSF
pT14SSG
PCR using following oligonucleotides:
5'-GGTGTTTTATGAAAAAGACAATCGCATTTCTTC-3'
3'-CCACAAAATACTTTTTCTGTTAGCGTAAAGAAG-5'
XbaI & MluI cleavage
XbaI
MluI
STII SD
STII
XbaI & MluI cleavage
MluI
pT14SSG
XbaI
ligation
pT14SSG-4T
PCR using following oligonucleotides:
5'-CAAATGCCCAAGCGACACCCCTGGGC-3'
3'-GTTTACGGGTTCGCTGTGGGGACCCG-5'
pT14SSG
-4T22Q
PCR using following oligonucleotides:
5'-TCTAGAGGTTGAGGTGTTTTATGA-3'
3'-AGATCTCCAACTCCACAAAATACT-5'
pT140SSG
-4T22Q

Fig. 6

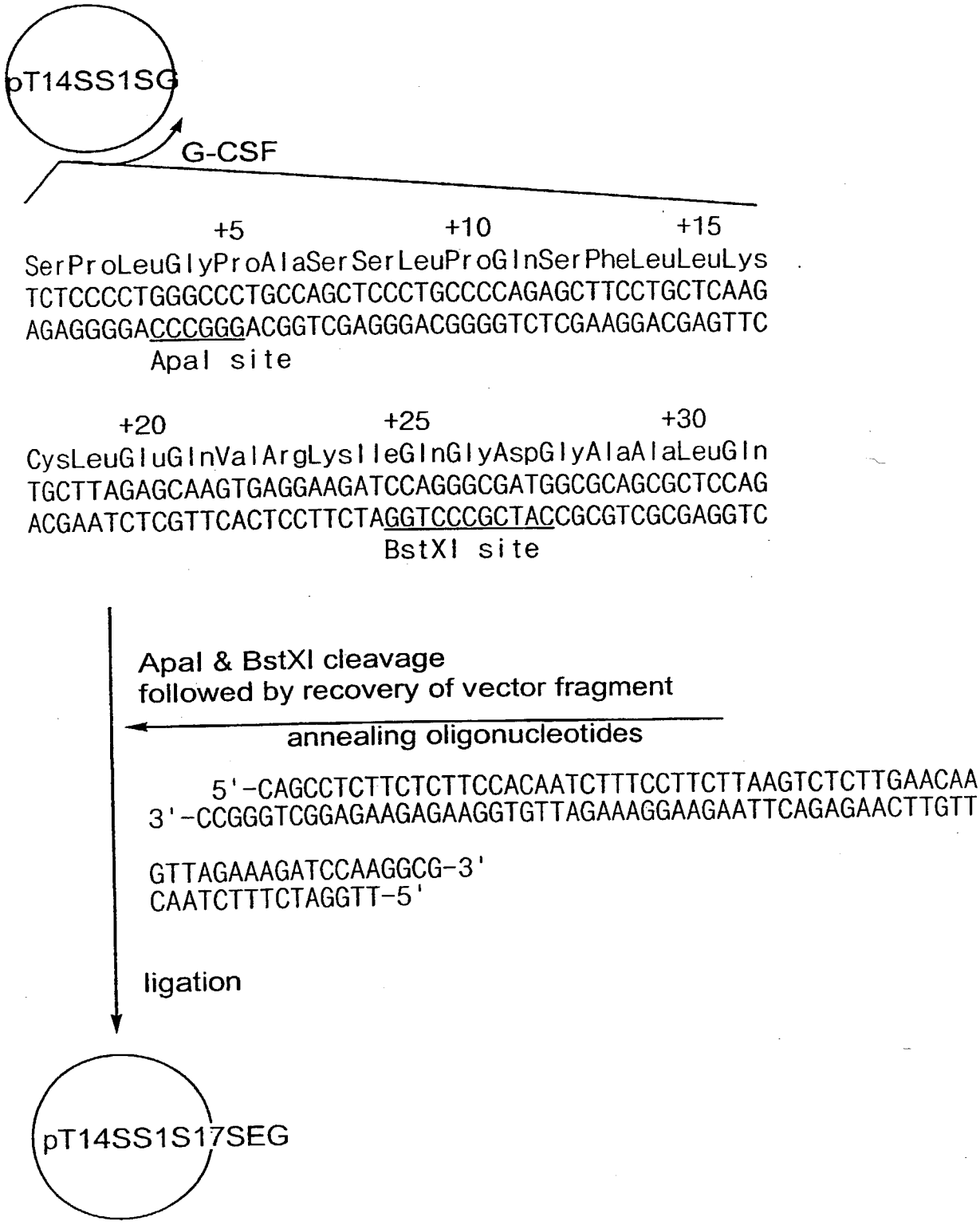

pT14SS1SG
G-CSF
+5
+10
+15
SerProLeuGlyProAlaSerSerLeuProGlnSerPheLeuLeuLys
TCTCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAG
AGAGGGGACCCGGGACGGTCGAGGGACGGGGTCTCGAAGGACGAGTTC
ApaI site
+20
+25
+30
CysLeuGluGlnValArgLysIleGlnGlyAspGlyAlaAlaLeuGln
TGCTTAGAGCAAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAG
ACGAATCTCGTTCACTCCTTCTAGGTCCCGCTACCGCGTCGCGAGGTC
BstXI site
ApaI & BstXI cleavage
followed by recovery of vector fragment
annealing oligonucleotides
5'-CAGCCTCTTCTCTTCCACAATCTTTCCTTCTTAAGTCTCTTGAACAA
3'-CCGGGTCGGAGAAGAGAAGGTGTTAGAAAGGAAGAATTCAGAGAACTTGTT
GTTAGAAAGATCCAAGGCG-3'
CAATCTTTCTAGGTT-5'
ligation
pT14SS1S17SEG

Fig. 7

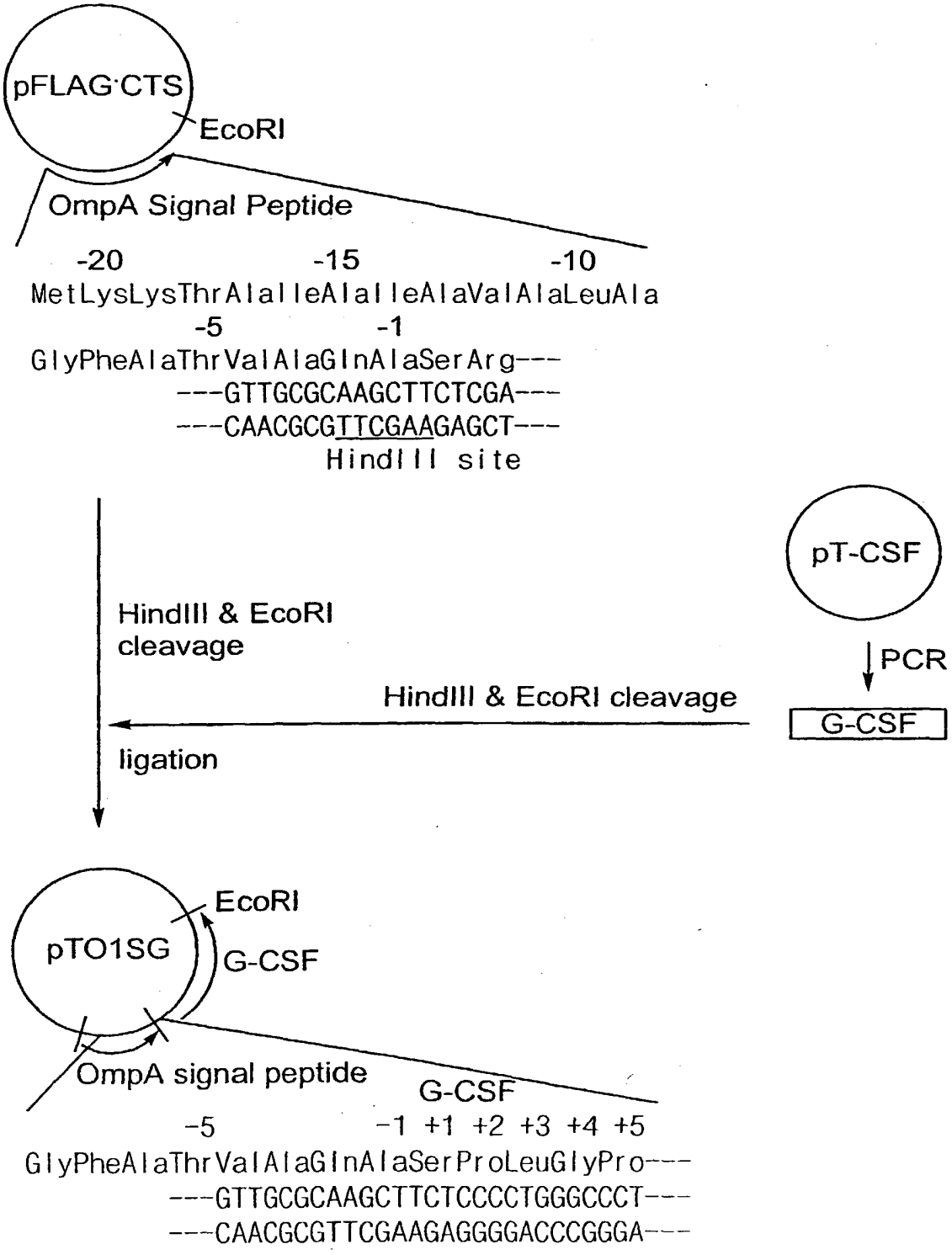

pFLAG·CTS
EcoRI
OmpA Signal Peptide
-20 -15 -10
MetLysLysThrAlaIleAlaIleAlaValAlaLeuAla
-5 -1
GlyPheAlaThrValAlaGlnAlaSerArg---
---GTTGCGCAAGCTTCTCGA---
---CAACGCGTTCGAAGAGCT---
HindIII site
pT-CSF
PCR
G-CSF
HindIII & EcoRI cleavage
HindIII & EcoRI cleavage
ligation
pTO1SG
EcoRI
G-CSF
OmpA signal peptide
G-CSF
-5 -1 +1 +2 +3 +4 +5
GlyPheAlaThrValAlaGlnAlaSerProLeuGlyPro---
---GTTGCGCAAGCTTCTCCCCTGGGCCCT---
---CAACGCGTTCGAAGAGGGGACCCGGGA---

Fig. 8

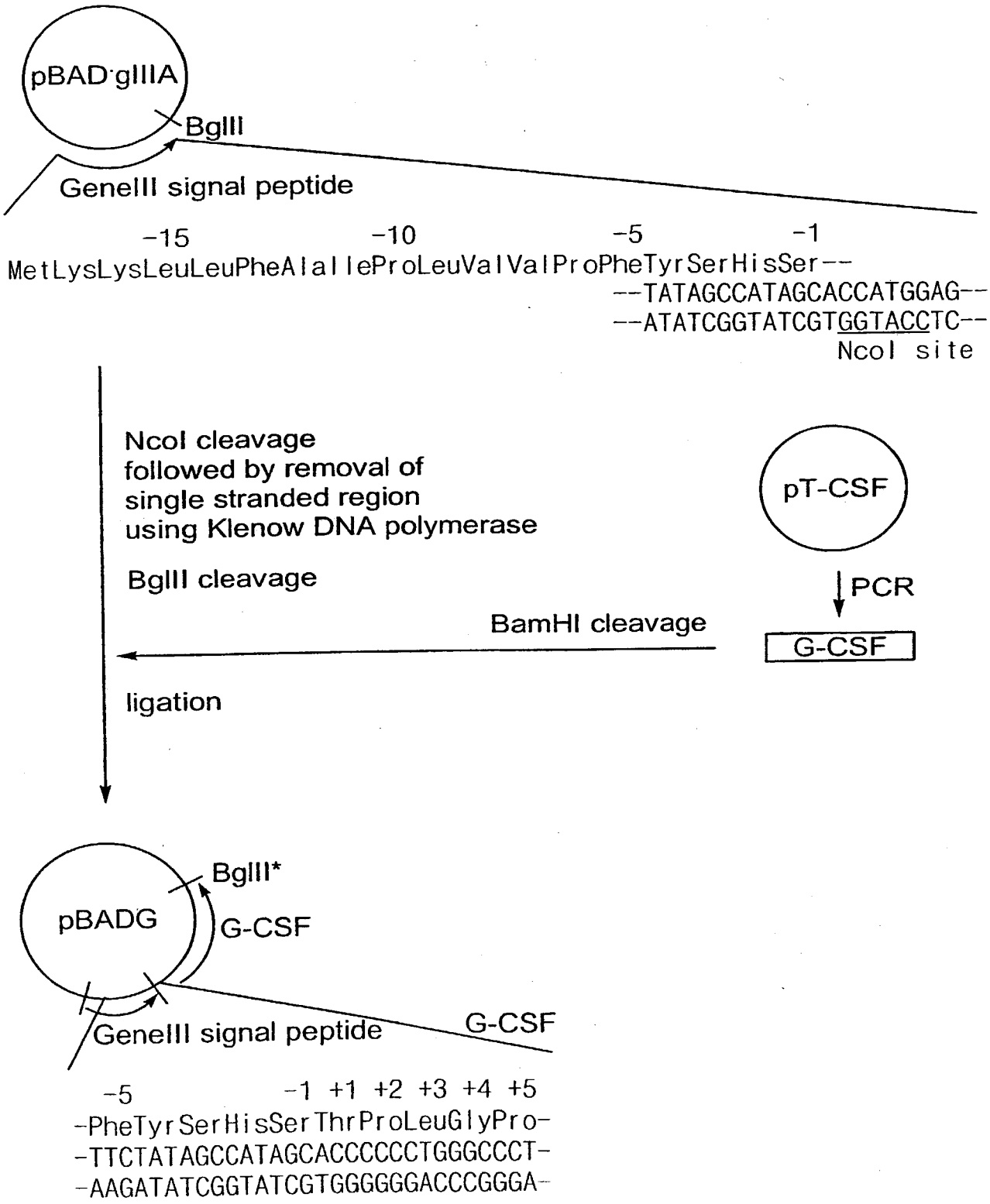

pBAD·gIIIA
BglII
GeneIII signal peptide
-15 -10 -5 -1
MetLysLysLeuLeuPheAlaIleProLeuValValProPheTyrSerHisSer--
--TATAGCCATAGCACCATGGAG--
--ATATCGGTATCGTGGTACCTC--
NcoI site
NcoI cleavage
followed by removal of
single stranded region
using Klenow DNA polymerase
BglII cleavage
pT-CSF
PCR
BamHI cleavage
G-CSF
ligation
pBADG
BglII*
G-CSF
GeneIII signal peptide
G-CSF
-5 -1 +1 +2 +3 +4 +5
-PheTyrSerHisSerThrProLeuGlyPro-
-TTCTATAGCCATAGCACCCCCCTGGGCCCT-
-AAGATATCGGTATCGTGGGGGGACCCGGGA-

Fig. 9

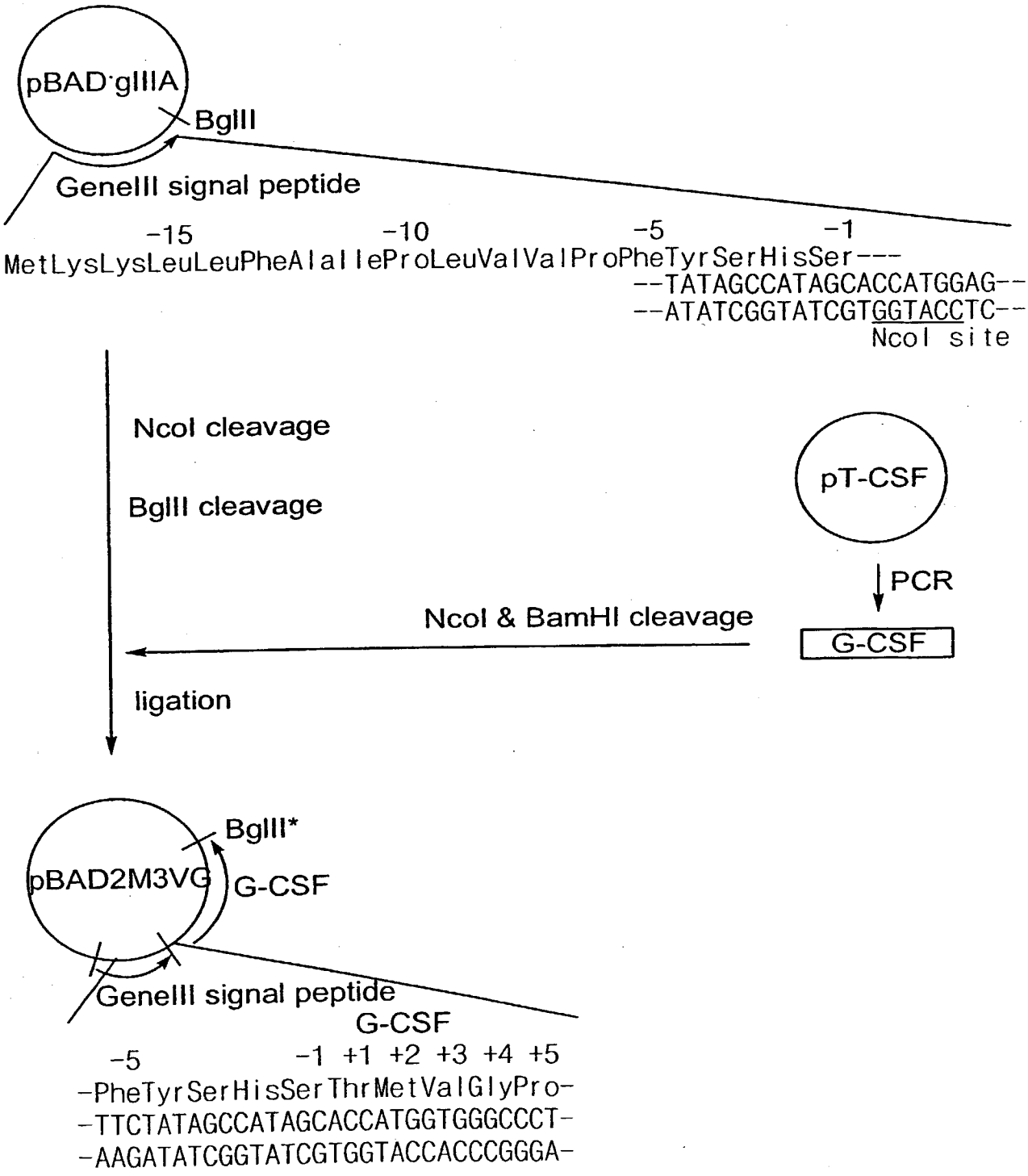

pBAD·gIIIA
BglII
GeneIII signal peptide
-15 -10 -5 -1
MetLysLysLeuLeuPheAlaIleProLeuValValProPheTyrSerHisSer---
--TATAGCCATAGCACCATGGAG--
--ATATCGGTATCGTGGTACCTC--
NcoI site
NcoI cleavage
BglII cleavage
pT-CSF
PCR
NcoI & BamHI cleavage
G-CSF
ligation
BglII*
pBAD2M3VG
G-CSF
GeneIII signal peptide
G-CSF
-5 -1 +1 +2 +3 +4 +5
-PheTyrSerHisSerThrMetValGlyPro-
-TTCTATAGCCATAGCACCATGGTGGGCCCT-
-AAGATATCGGTATCGTGGTACCACCCGGGA-

Fig. 10A

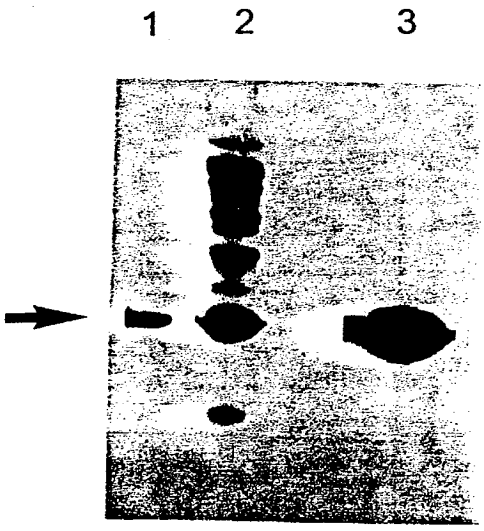


Fig. 10B

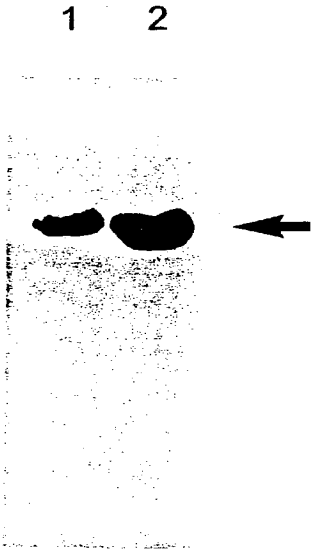
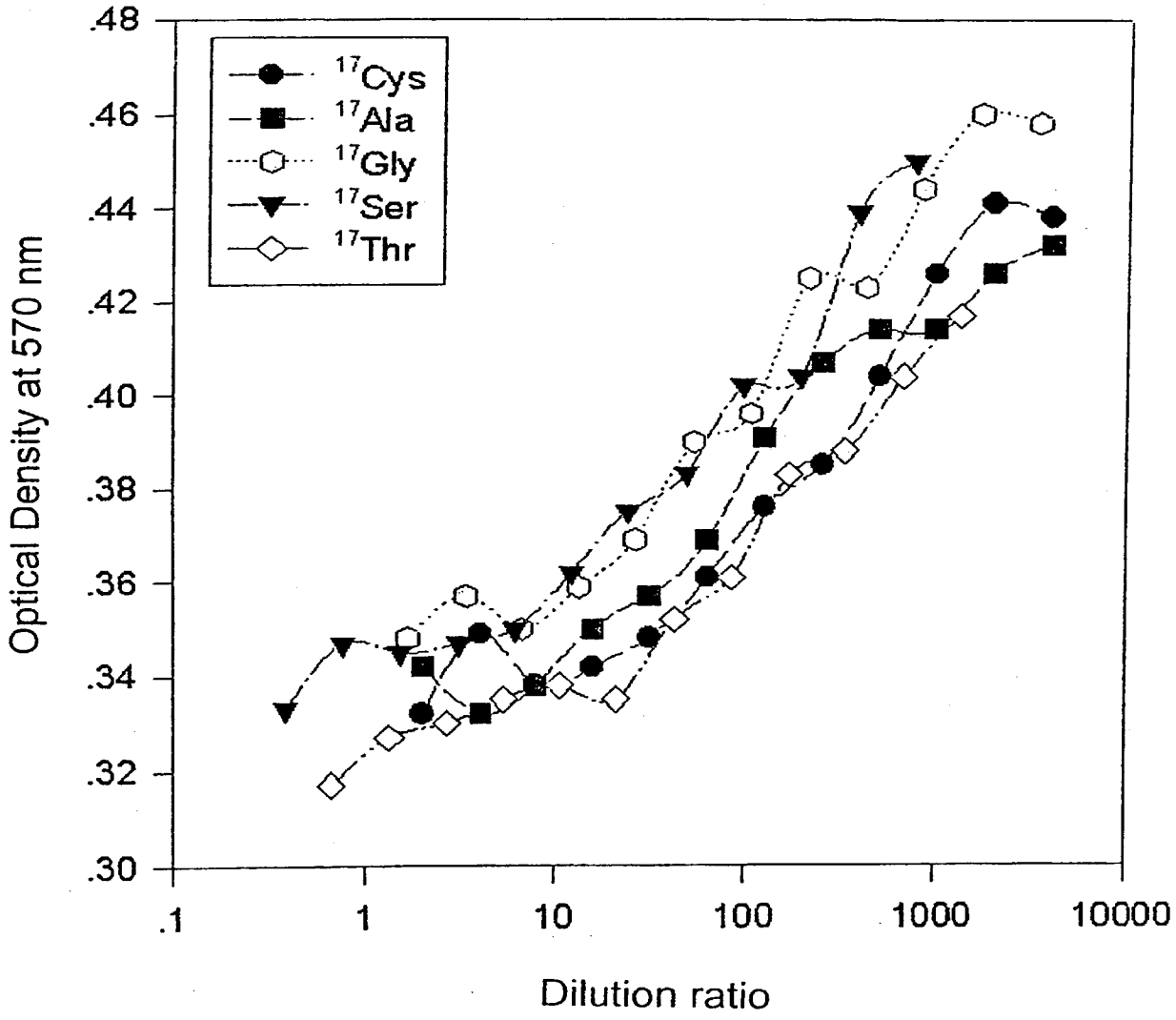


Fig. 11


.48
.46
.44
.42
.40
.38
.36
.34
.32
.30
Optical Density at 570 nm
^{17}Cys
^{17}Ala
^{17}Gly
^{17}Ser
^{17}Thr
.1
1
10
100
1000
10000
Dilution ratio